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Karl Persson

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Karl Persson

Supervisors:

Assoc. Prof. Mattias Thelander, SLU, Department of Plant Biology and Forest Genetics

Dr. Tom Martin, SLU, Department of Plant Biology and Forest Genetics

Examiner:

Senior Research Lecturer. Jens Sundström, SLU, Department of Plant Biology and Forest Genetics

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1. Abstract

The knowledge of programmed cell death (PCD) in plants is limited. The gene family of caspases in animals is known to be important for the execution of PCD. Putative homologues to caspases have been found in plants and are referred to as metacaspases. The model moss *Physcomitrella patens* is known to possess a high frequency of homologous recombination, which can be exploited for the generation of targeted gene knockout mutants. The female reproductive organ in *P. patens* shows rapid cell degradation during its maturation. This cell degradation is believed to be caused by PCD, and is therefore used as a model to study the process. A new metacaspase 5 knockout construct was successfully produced during this project, which, together with a metacaspase 3 construct received from a collaborator, was transformed into moss to generate two metacaspase single knockout lines and four double knockout lines.

2. Table of contents

3. Introduction

The process in which cells commit suicide through programmed cell death (PCD) is essential for normal development of a multicellular organism. There are two major types of PCD in animals: apoptosis and autophagy-dependent. The morphologies associated with apoptosis are chromatin condensation, shrinkage of plasma membranes, DNA degradation and cellular fragmentation into smaller parcels called “apoptopic bodies”, which are engulfed by macrophages and degraded by lysosomal enzymes (van Doorn, 2011; van Doorn et al. 2011; Lam, 2008; Vercammen et al., 2007). During autophagic PCD the cytoplasmic material in the cell is degraded by the cells own lysosomal activity. Autophagy is also a process for recycling of cellular components in cells that are not undergoing cell death. Cells can also die by necrosis, in which the cell swells up and bursts in a highly uncontrolled manner (van Doorn et al., 2011).

Plants like animals need PCD for several purposes. These include organ development, germination, senescence, stress responses (salt and temperature changes) and pathogen defense responses (Hara-Nishimura and Hatsugai, 2011; Reape and McCabe, 2010; Williams et al., 2008). Apoptosis is not present in plant cells because they are separated from neighboring cells by a thick cell wall and there are also no phagocytic cells (Lam, 2008. van Doorn et al. 2011).

Little is known about the mechanisms of PCD in plants, but it is believed that some of the knowledge from animal models also can be applied on plants. Caspases are a family of proteases known to be crucial for the execution of PCD in animals (Coll et al., 2011; Coll et al., 2010; Tsiatsiani et al., 2011; Uren et al., 2000; Woltering 2010). Based on predicted structural similarities to the active site of animal caspases, proteins referred to as metacaspases were found in plants (Uren et al., 2000). Other genes thought to be important for the PCD mechanism in plants are vacuolar processing enzymes (VPE), Proteasome subunit PB1 and the protease target Tudor Staphylococcal Nuclease (TSN; Frey et al., 2010; Sundström et al., 2009). Genes involved in autophagy are present in plants and referred to as ATG genes (Mitou et al., 2009).

Metacaspases are a group of Cysteine- dependent proteases that have been found in fungi, protozoa and plants (Vartapetian et al., 2011). Plant metacaspases, which form two subgroups referred to as type I and type II, are structurally similar to animal caspases, a family of proteases known to be crucial for the execution of PCD (Coll et al., 2011; Coll et al., 2010; Tsiatsiani et al., 2011; Uren et al., 2000; Woltering 2010). Metacaspases belonging to the type I group have an N-terminal prodomain that contains a proline rich repeat motif. In plant type I metacaspases there is also a domain with a zink finger motif. Type II metacaspases (not present in fungi and protozoa) lack the prodomain, but have a linker region between a large and small subunit. Metacaspases have a His-Cys catalytic dyad where the active site is predicted, the Cys residue is believed to be the nucleophile that acts on the substrate (Tsiatsiani et al., 2011).

The number of metacaspase genes varies a lot between different organisms. Gene redundancy makes it difficult to study knockout lines when only one of several metacaspase genes has been knocked out (Tsiatsiani et al., 2011).

To identify the key components of the PCD pathway in plants, a simple model is desired. The model should be easy to manipulate by gene targeting, have a low variation and be easy to study phenotypically under *in vivo* conditions. Canal cell clearance during female reproductive organ (archegonia) development in the moss *Physcomitrella patens,* which belong to the lineage bryophytes, meet these criteria.

The Bryophytes are thought to have diverged from land plants ~450 million years ago and *P. patens* was the first non-vascular plant to have had its genome sequenced (Rensing et al., 2008). It has a simple morphology, but is still highly regulated by environmental cues, genetic programs and hormones by mechanisms which are believed to be at least partly shared with higher plants (Prigge and Bezanilla, 2010). *P. patens* has often been used in studies on the evolution of plant physiology by comparisons to *Arabidopsis thaliana* and other seed plants (Rensing et al., 2008; Cove et al., 2006).

The production of loss-of-function mutants can give insights in what processes a protein of interest is involved in by studying the resulting phenotypes. It is relatively easy to target genes in *P. patens* as it exhibits a high frequency of homologous recombination (HR) that is a naturally occurring process. Gene knockout mutants are easily produced (Frank et al., 2005; Kamisugi et al., 2005; Kamisugi et al., 2006) as a specifically designed DNA fragment will align with the targeted locus and replace the allele by HR (figure 4, step 5). The life cycle of *P. patens* furthermore has haploid dominance, making the effects of gene mutations such as knock-outs discoverable (Cove, 2005).

P. patens is a monoecious moss, having both sexual organs present at the same plant (Schaefer and Zrÿd, 2001). When the moss female sexual reproductive organ (archegonium) matures, a central cell file in its neck portion is degraded to allow sperms to access the egg protected in a deep cavity (figure 1, Landberg et al., 2012). It is believed that the clearance of this cell file depends on readily observable PCD representing a good model for the study of PCD in plants.

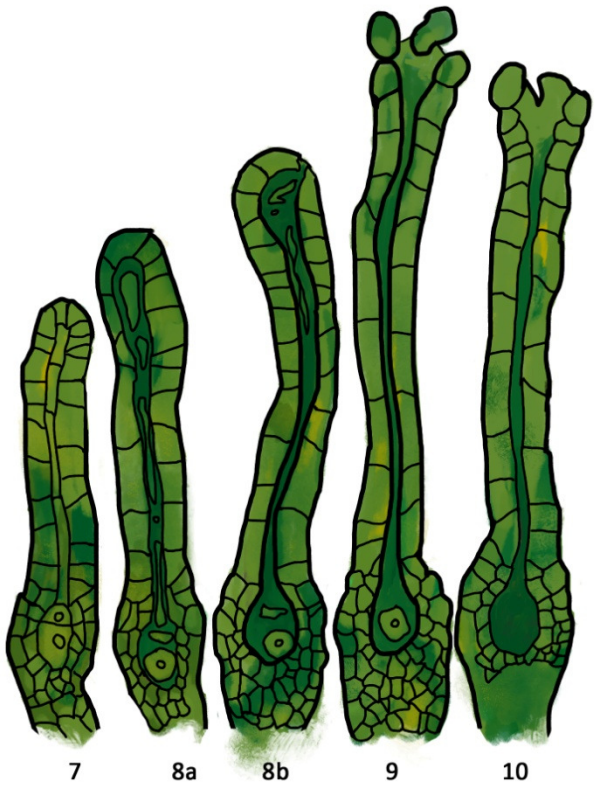

Figure 1. The development of *P. patens* archaegonia. In stage 7 there are two short and two long inner canal cells. The four canal cells degrade in stage 8a and b, the tip burst open leaving the egg cell accessible for fertilization (stage 9). In stage 10 the outer neck cells content are degraded. Redrawn from Landberg et al. (2012).

3.1 Aim of project

The aim of the project was to study the role of metacaspases during canal cell degradation in moss archegonia by generating metacaspase knockout mutants. *P. patens* has two type I and four type II metacaspases (Tsiatsiani et al., 2011; figure 2). One gene of each type has previously been knocked out without any apparent effects on archegonia development (Martin and Thelander, unpublished). Since this may well be due to functional redundancy, the project aimed to generate one type I double mutant line and one type II double mutant. In the latter case, the type II gene *PpMC5* showing most similarity to the type II gene already knocked out (*Ppmc2*) was targeted (figure 2). The type I *PpMC3* knockout construct used to transform the pre-existing *Ppmc1* line was received from collaborator Sabina Vidal (University of Montevideo, Uruguay), while the type II *PpMC5* knockout construct used to transform the pre-existing *Ppmc2* line was built during the project. During the short timeframe given for the project, genotyping of putative *Ppmc3* and *Ppmc5* knockouts was not achieved. Instead, genotyping of *Ppatg2* knockouts produced by Tom Martin was preformed.

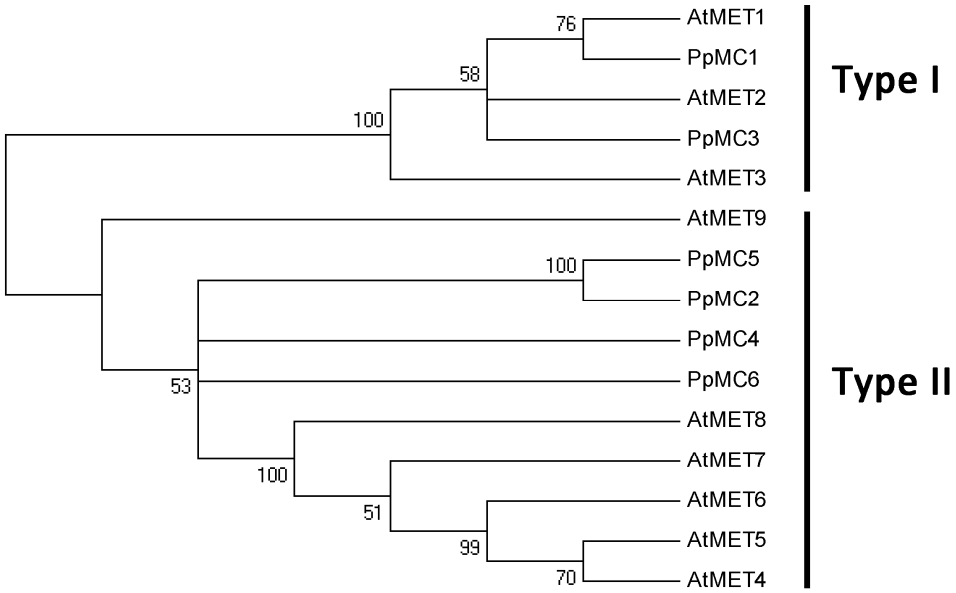

Figure 2. Phylogenetic tree depicting the relationship of type I and II metacaspases in *Physcomitrella patens* and *Arabidopsis thaliana*, bootstrap values are shown.

4. Methods

4.1 Primer design for PCR amplification, sequencing and genotyping

Primers for amplification and sequencing were designed using web recourses primer 3 (http://frodo.wi.mit.edu/) and the cosmoss (http://www.cosmoss.org/). The phylogenetic tree showing the distribution of metacaspases in *Arabidopsis thaliana* and *Physcomitrella patens* was produced using amino acid sequences aligned by ClustalW and a neighbor joining method in MEGA 5 with 1000 bootstrap interations.

The primers were designed for amplification of the *PpMC5* 5' flanking region (5PFR) and 3' flanking region (3PFR) from an extracted genomic template. The desirable fragment length of 5PFR is 1127 and 3PFR is 1158 before enzymatic trimming (table 1). An overview of the cloning procedure can be seen in figure 4.

Primers used for sequencing the completed *PpMC5* construct was designed to sequence both the flanking regions, all primers except 5PFR forward was made in duplicates, in the case that one sequencing reaction would not succeed because the primer.

Region	Number	Type	Annealing	Primer
5PFR	1	Amplification	Forward	CGC TAT GTC GAC AAT TCG AAT GCG TTA ATT T
5PFR	2	Amplification	Reverse	TCA ACC GAA CGT CTT CAG TG
3PFR	3	Amplification	Forward	TTG ACT TGC AAA GCA GT
3PFR	4	Amplification	Reverse	TGA GCT CCA AGG AGC GAA GCA GTT CGC TAT
5PFR	5	Sequencing	Forward	GTT TTC CCA GTC ACG ACG TT
5PFR	6	Sequencing	Reverse	TCT ACG TGT TCC GCT TCC TT
5PFR	7	Sequencing	Reverse	CCA TAA AAC CGC CCA GTC TA
3PFR	8	Sequencing	Forward	TGC AGG TCA AAC CTT GAC AG
3PFR	9	Sequencing	Forward	CAG TGA CGA CAA ATC GTT GG
3PFR	10	Sequencing	Reverse	TGT GGA ATT GTG AGC GGA TA
3PFR	11	Sequencing	Reverse	GAG TCA GTG AGC GAG GAA GC
5' ATG2	12	Genotyping	Forward	GGC TCA TAG GCC TTT AAC TTG
5' ATG2	13	Genotyping	Reverse	CCA GTC TTT ACG GCG AGT TC
3' ATG2	14	Genotyping	Forward	TTC GCT CAT GTG TTG AGC AT
3' ATG2	15	Genotyping	Reverse	TCG TGA AGC TCG CTA TTC CT
ATG2 gene	16	Genotyping	Forward	GCG ATG GGG AGA AAC AGA TA
ATG2 gene	17	Genotyping	Reverse	AAA GTT TCA GGC CAA CAT GC

Table 1. Primers used for amplification of the flanking regions and for sequencing

4.2 PCR amplification and purification of the *PpMC5* 5´and 3´genomic flanking regions

For every reaction a mixture of 18 µl H_2O, 5 µl Phusion HF Buffer, 0.5 µl dNTP (10 mM), 0.5 µl (10 µM) of each primer (table 1), 1 µl genomic template and 0.2 µl Phusion enzyme (Thermo Scientific) was used. The PCR program started with 98°C for 30 seconds, then for 35 cycles the PCR was run at 98°C for 10 seconds, 60°C for 20 seconds and 72°C for 1 minute. After the 35 cycles the temperature was set to 72°C for 5 minutes and put at hold on 12°C. The PCR products were tested on an agarose gel to check that the fragments were of the correct sizes prior to the removal of nucleotides and primers using the QIAquick PCR purification protocol.

4.3 Restriction Enzyme digestions

The 5PFR fragment was trimmed and the pMT123 vector was linearized using restriction enzymes *Sal*I/*Hind*III (New England Biolabs). *Sal*I was used to cut 5PFR and pMT123 vector followed by a basic ethanol precipitation (1/10 volume 3 M sodium acetate and 2.5 volume 95 % ethanol). The redissolved DNA was digested with *Hind*III and heat inactivated according to the company recommendations.

Screening for positive 5PFR insertion into pMT123 was made using enzymes *Bam*HI, *Sap*I, *Hinc*II and *Bgl*I. In a reaction volume of 10 µl for each reaction, 3 µl extracted plasmid was digested with 0.5 µl enzyme. One positive plasmid was given the name pKP1 that together with TOPO cloned 3PFR was cut using restriction enzymes *Spe*I and *Sac*I (Fermentas). The

enzyme digested fragments were gel extracted using the protocol provided with the QIAquick gel extraction kit (QIAGEN). Screening for a positive 3PFR insertion into pKP1 was performed using restriction enzymes *Psi*I, *Bpu*10I and *Bst*BI. One clone proven to contain the desired 3PFR insert was given the name pKP2.

The *PpMC3* and *PpMC5* knockout vectors were linearized using *Sac*I/*Kpn*I (New England Biolabs) and *Sac*I/*Sal*I (Fermentas), respectively, in a reaction volume of 350 µl with 300 µl (0.5 µg/µl *PpMC3,* 1.5 µg/µl PpMC5) pure plasmid, 8 µl of each enzyme, and (1X) buffer. The digests were tested on a gel to confirm correct fragment sizes prior to transformation into moss.

4.4 Ligation and transformation into competent *E. coli* cells

Vectors and inserts were ligated in a 1:3 ratio using 2 µl T4 ligase (Fermentas) together with buffer and distilled H_2O to a total reaction volume of 20 µl. The reaction mixtures were incubated at 16°C over night in darkness.

For transformation, 10 µl ligation mix was added to 50 µl competent Top10 cells. The tubes were incubated on ice for 10 minutes and then heat shocked for 60 seconds in a 42°C water bath. 700 µl liquid LB media was added to the tubes and put on a shaker for 1 hour at 37°C, 220 rpm. 50 µl was plated out on a LB Ampicillin (100 µg/ml) plate. The remaining bacteria were pelleted by centrifugation in 4200 x g for 5 minutes. The supernatant was decanted and the pellet was gently resuspended by pipetting and plated out on Ampicillin (100 µg/ml) plates. The plates were incubated in 37°C overnight. For plasmid mini preps single colonies were used to inoculate 4 ml LB Ampicillin (100 µg/ml). The bacteria cultures were grown overnight in 37°C at 220 rpm.

The 3PFR PCR fragment was cloned using the pCR™II-Blunt-TOPO® vector following the recommendations from Invitrogen.

The GeneJET plasmid miniprep kit (Fermentas) was used for all the analytical plasmid extractions, all plasmids were eluted in 50 µl. The plasmid Maxi prep (QIAGEN) was used to extract a greater amount of the *PpMC3* and *PpMC5* knockout constructs. The *PpMC5* plasmid was resuspended in Tris-HCl buffer and *PpMC3* in H_2O. The DNA concentrations were measured using nanodrop (ND-1000). The purified pKP2 plasmid was sent for sequencing using the sequencing primers in table 1.

4.5 Moss transformation

6 different knockout lines were produced during the project, as illustrated in figure 3. Protoplasts were isolated according to Grimsley et al. (1977) by adding 6 day old proteonema tissue (wildtype, *Ppmc1* knockout and *Ppmc2* knockout) with 1% w/v Driselase (SIGMA) in 8.5 % D-mannitol for 35 minutes. The digested plant material was passed through a 100 µm filter. The digested moss was then incubated with the Driselase for another 10 minutes, and passed through a fine 50 µm filter. The protoplasts were centrifuged at 78 x g for 5 minutes, with the brake turned off. The pellet was washed twice with 8.5 % D-mannitol to the original density.

The number of healthy protoplasts was estimated by microscopy using a Burcher chamber. The protoplasts were pelleted through centrifugation at 78 x g for 5 minutes. The protoplasts were resuspended in MMM (Schaefer et al., 1991) to a density of 1.2 x 10^6 protoplasts/ml. Knockout constructs for *PpMC3* and *PpMC5* were was aliquoted to different tubes (10-30 µg DNA) together with 300 µl protoplast solution. The protoplasts were heat shocked for 5 minutes (*Ppmc3* knockout) and 10 minutes (*Ppmc5* knockout) at 45°C and allowed to cool down to room temperature (20°C) for 10 minutes, with a gentle mix every second minute.

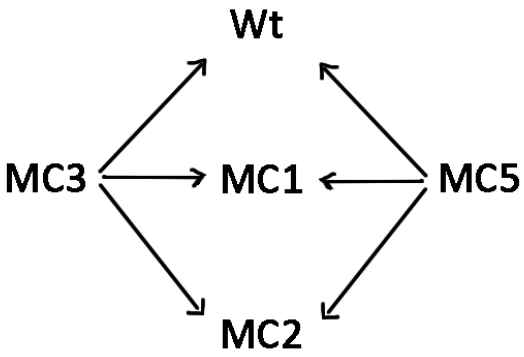

Figure 3. Transformations to generate 6 different knockout lines were done during the project; three *Ppmc3* knockouts (*Ppmc3*; *Ppmc3/1*; *Ppmc3/2*) and three *Ppmc5* knockouts (Ppmc5; Ppmc5/1; Ppmc5/2).

The transformation mix was progressively diluted with regeneration media to a total volume of 7 ml. The protoplasts were incubated for 15 hours in the dark in room temperature. Without mixing the sedimented protoplasts, 4 ml of the regeneration media was removed. The protoplasts were resuspended in the remaining 3 ml of regeneration media and a 3 ml top layer of regeneration media (42°C) was added. 2 ml of the mix was transferred to 3 cellophane overlaid regeneration plates. The protoplasts were grown at 25°C in a growth chamber (Sanyo MLR-350) under constant white light (30 $\mu E \cdot m^{-2}s^{-1}$) from the sides for 6 days.

After 6 days the transformed moss was transferred to BCD plates supplemented with 5 mM ammonium tatrate (Thelander et al., 2007) containing hygromycin B (30 µg/ml) for selection. After 13 days the putative *Ppmc3* knockout transformants were transferred off selection for 13 days and then transferred back to selection.

4.6 Genotyping by PCR

A small piece (0.3 cm^2) of plant material from putative *Ppatg2* knockout candidates was extracted using the QuickExtract™ plant DNA Extraction Solution protocol (epicentre). The extracted DNA template was amplified using the Phusion enzyme protocol (section 4.2) with primers 12-17 (Table 1). The PCR products were tested on an agarose gel for DNA fragments of the desired lengths.

5. Results

5.1 The *PpMC5* knockout construct – overview of cloning strategy

Figure 4 shows an overview of the cloning strategy followed to produce the *PpMC5* knockout construct. The plasmid vector pMT123 used contains a resistance cassette for Ampicillin, used for selection in bacteria, and a Hygromycin B resistance gene driven by the NOS1 promoter, for selection in moss. From a *P. patens* wild type genomic template, the 5' flanking region (5PFR) and the 3-flanking region (3PFR) were amplified with primers adding restriction sites allowing insertion on either side of the hygromycin resistance cassette by sticky end ligation. The completed knockout construct was released by restriction digestion and used for transformation of wild type as well as the *Ppmc1* and *Ppmc2* knockout lines (figure 4).

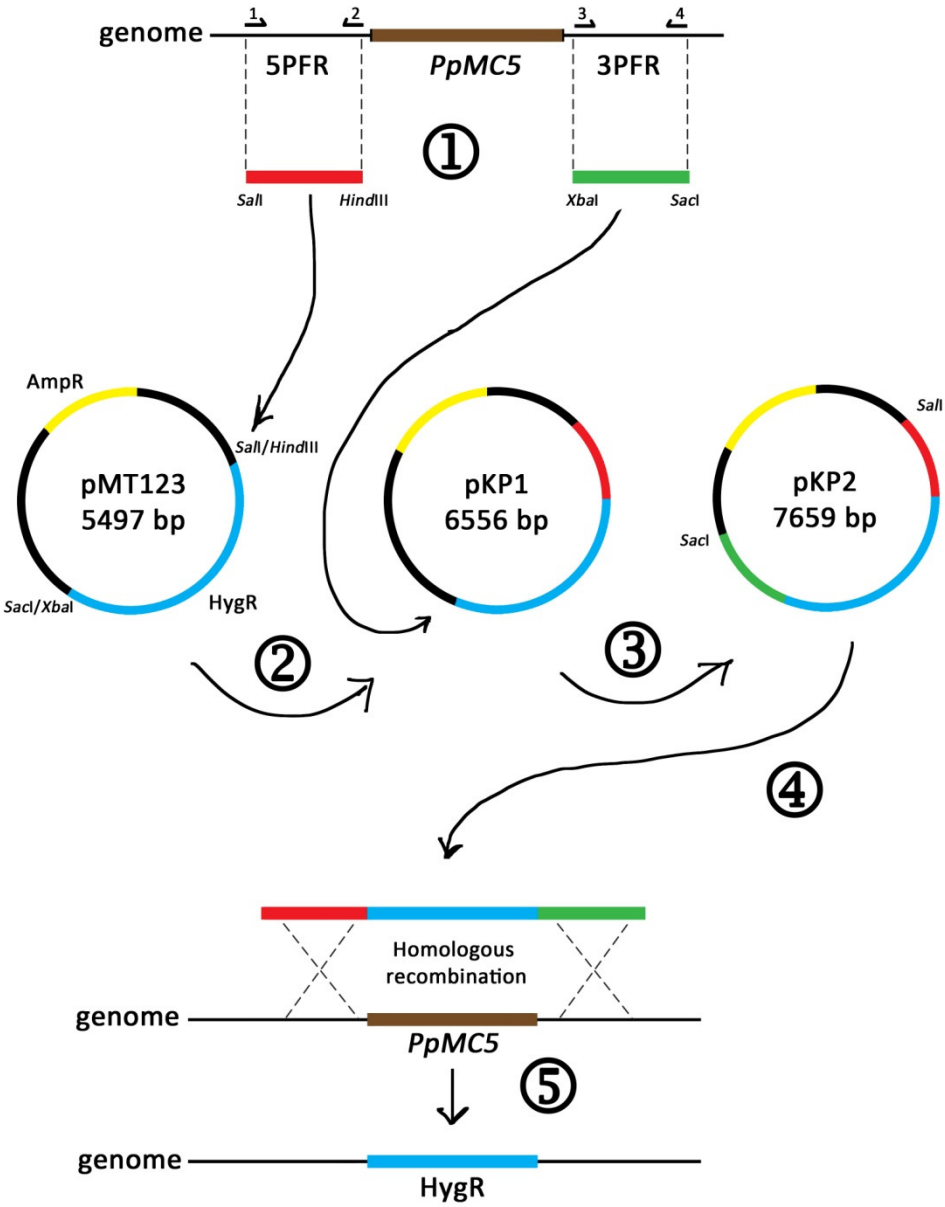

Figure 4. Overview of cloning strategy. The flanking regions on both sides of the gene (brown) were amplified (step 1) using primer pairs 1/2 and 3/4 and inserted into the plasmid vector (steps 2 and 3). The completed construct was linearized (step 4), transformed into moss and hopefully integrated into the genome by HR (step 5).

5.2 PCR of the *PpMC5* flanking regions

The *PpMC5* 5’ and 3’ flanking regions were PCR amplified using the primer pairs 1/2 and 3/4 (table 1; figure 4), respectively, and *P. patens* genomic DNA as the template. The correct sizes of the products were confirmed by agarose gel analysis (figure 5).

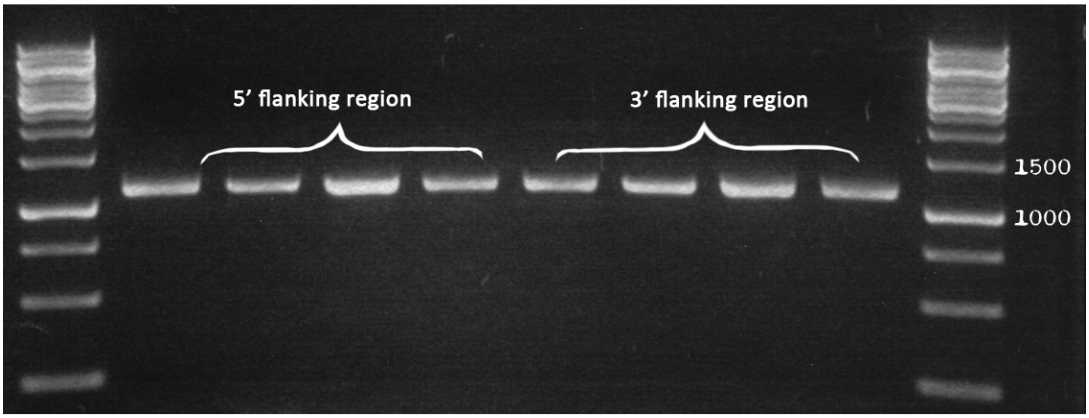

Figure 5. The PCR amplified *PpMC5* 5’ and 3’ flanking regions visualized on an agarose gel to confirm desired lengths of 1127 bp and 1158 bp, respectively.

5.3 Cloning of the 5’ flanking region

The vector pMT123 and the 5PFR fragment were both cut with restriction enzymes *Sal*I/*Hind*III and ligated. In the first attempt to transform competent cells with the ligation mix, one bacterial colony was produced while a second transformation yielded many colonies. Plasmids extracted from a total of 17 clones were screened by enzyme digestion with *Bam*HI to test for insertion of the 5PFR insert (figure 6). The plasmids carrying the desired insert should have three defined bands compared to the original vector without an insert that only will be cut on two restriction sites, thus revealing two bands on a gel. All plasmids were also tested with *Sap*I, but because of inefficient enzyme activity, no results that could be interpreted were obtained.

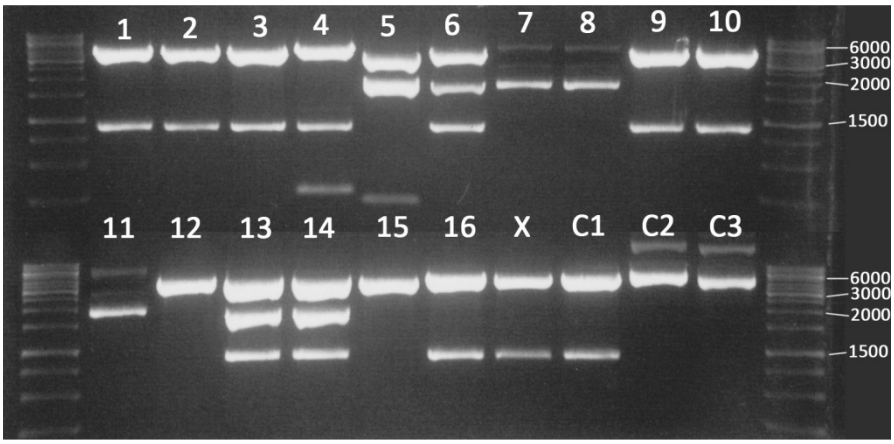

Figure 6. *Bam*HI test digestions. The colonies picked from the second transformation are termed 1-16, and the single colony from the first transformation is termed X. The controls are digested pMT123 plasmid (C1), circular pMT123 plasmid (C2) and circular X plasmid (C3). The expected pattern from the desired ligation product is three fragments with the sizes 1786, 888 and 3882 bp, compared to the cut vector (C1) that should have two bands with the lengths 888 and 4609 bp. The result suggests three candidates that may contain the 5PFR insert; clones 6, 13 and 14.

The potentially positive clones 6, 13 and 14 together with clones 1 and X, that previously gave negative results, were further test digested with restriction enzymes *Hinc*II (figure 7) and *Bgl*I (figure 8). Results support that clones 13 and 14 represent the desired ligation product. The *Bgl*I digest gave bands of the correct sizes, except for one extra band that have the approximate size of the undigested vector control (C2). Since the extra band was also present in the digested vector control (C1), it indicates that the *Bgl*I digest was incomplete.

The *Sap*I digestion which previously failed to give informative results was also repeated for the same 5 clones (figure 9) and now gave weak bands for clones 13 and 14 that could indicate that they contain the desired insert. A bright, broad band could be seen that indicates that the digest was not complete, leaving most plasmids uncut. The size of the bands were however hard to measure since the gel was ran in a U-shaped manner.

In summary, clone number 13 was concluded to represent the desired ligation product and was therefore given the name pKP1.

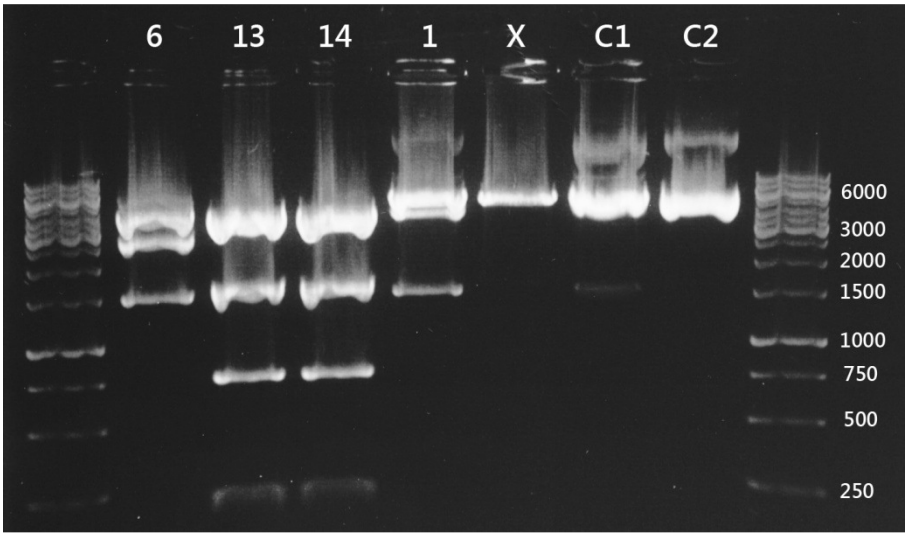

Figure 7. *Hinc*II test digestion. Expected fragment lengths for the desired ligation product are 164, 759, 1622 and 4011 bp. Cut vector control (C1) should have the lengths 1486 and 4011. A very faint band can be seen on C1 corresponding to 1486 bp. C2: Uncut vector control. Results support that clones 13 and 14 are positive.

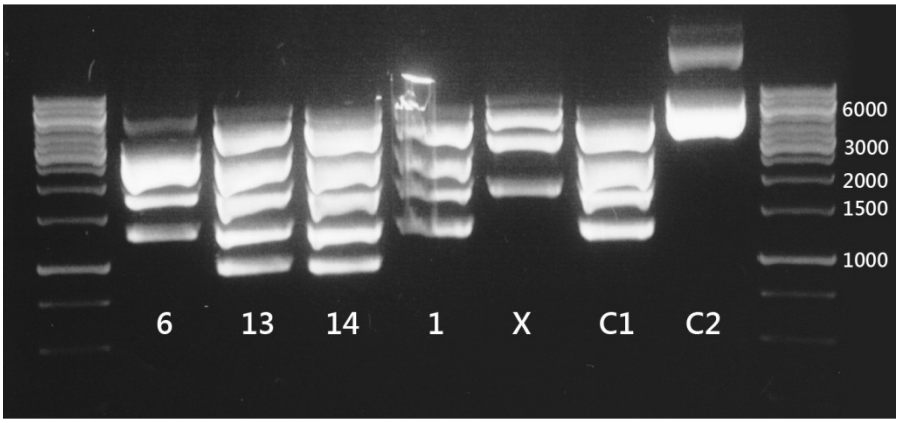

Figure 8. *Bgl*I test digestion. Expected lengths for the desired ligation product are 938, 1267, 1780 and 2571 bp. The cut vector control (C1) is expected to have the fragment lengths 1780, 1267 and 2450 base pairs. The samples 13 and 14 have 5 bands, 4 of which support that they contain the correct insert and one likely to depend on incomplete digestion. C2: Uncut vector control.

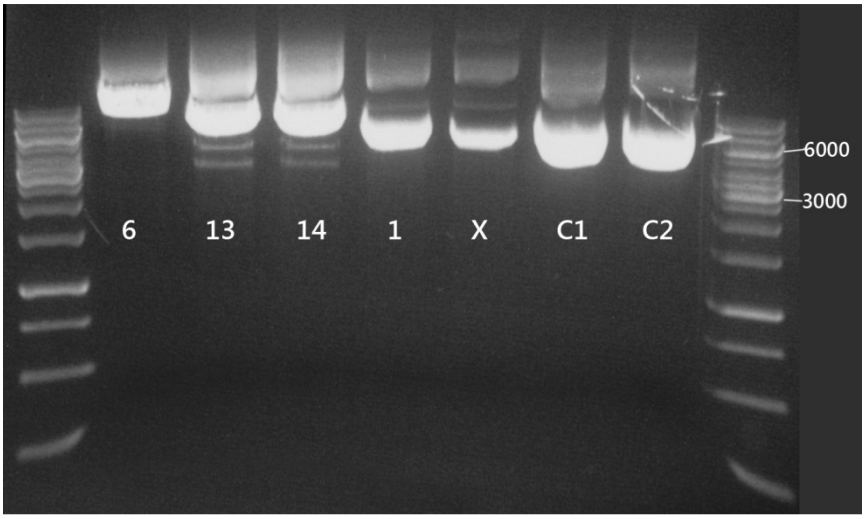

Figure 9. *Sap*I test digestions. The *Sap*I digested plasmids containing the 5PFR is expected to have two bands corresponding to the lengths 2813 and 3743 bp, compared to the cut vector control (C1) that will only be cut on one site generating a linearized plasmid with only one defined band. C2: Uncut vector control.

5.4 Cloning of the 3' flanking region

The pKP1 plasmid was linearized using restriction enzymes *Xba*I/*Sac*I and the 3PFR fragment was digested with the same enzymes to produce compatible ends for ligation. The resulting linear DNA was separated on an agarose gel where after desired fragments were excised and extracted to clean them from the short DNA fragments cut out of the vector and ends trimmed away, respectively (figure 10).

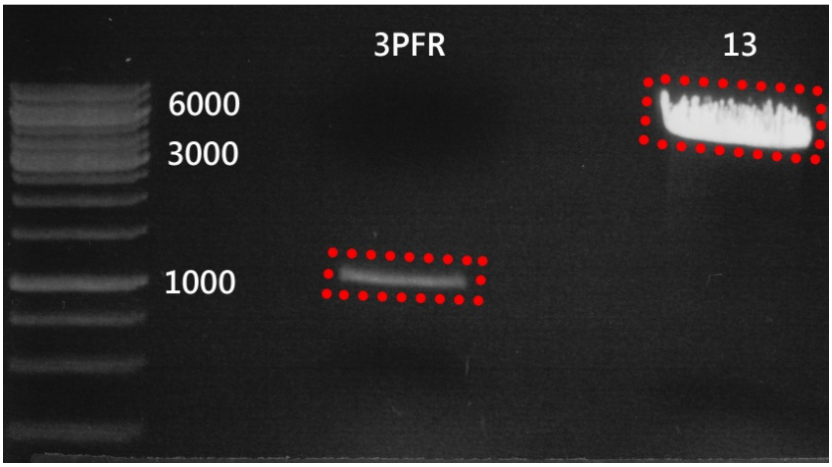

Figure 10. The 3PFR PCR product and pKP1, both digested with *Sac*I and *Xba*I and loaded on a gel. The expected fragment sizes are 1126 bp for the 3PFR fragment and 6522 bp for the pKP1 vector. The red dotted lines indicate bands that were cut out and gel purified.

The gel extracted pKP1 plasmid and 3PFR fragment were ligated and repeatedly transformed into competent *E. coli* cells but no transformants were obtained. The concentration of the 3PFR fragment was therefore suspected to be too low following gel extraction. This was repeated with a higher concentration of amplified 3PFR fragment which was again trimmed with *Sac*I/*Xba*I, this time without subsequent gel purification, and ligated with gel purified pKP1. The transformation was once again unsuccessful though with only a few transformants obtained that all gave negative results following test digests.

To really assure high quantity and quality of the insert in the ligation mixture the 3PFR PCR fragment was next blunt cloned into the commercial pCR™II-Blunt-TOPO® cloning vector. The cloning reaction yielded many bacterial colonies. Plasmid DNA was extracted from five clones and test digested with *Eco*RI to verify that the insert of the correct length had been cloned (figure 11). Plasmid DNA from TOPO clones 1 and 5 were then digested with *Xba*I/ *Sac*I to release the 3PFR fragment which was separated from the vector fragment on a gel, excised and purified (figure 12).

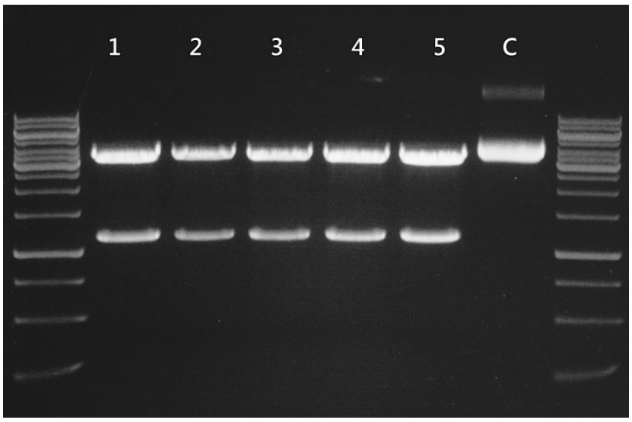

Figure 11. *Eco*RI test digestion. The *Eco*RI digest gave a positive result for all the 5 extracted plasmids with a band of 1177 bp for the cloned insert and 3500 bp for the vector. C1: clone 1 uncut.

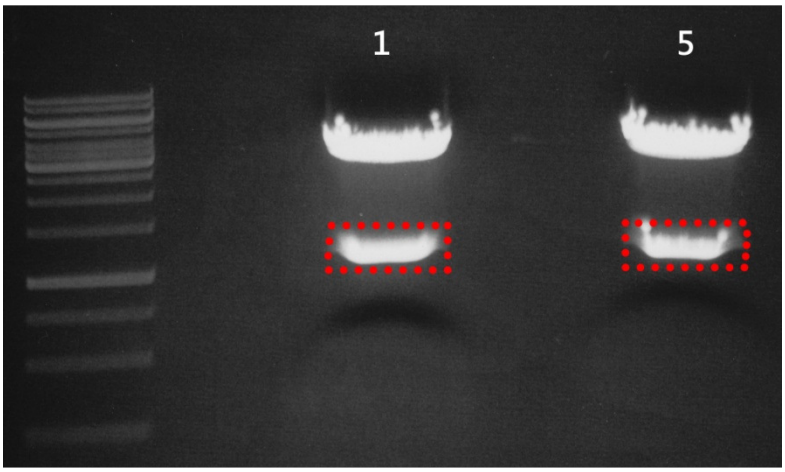

Figure 12. The *Sac*I and *Xba*I digested TOPO clones were loaded on a gel and the desired bands (marked in red) of 1126 bp were extracted.

The gel purified 3PFR fragment from TOPO clone 1 was ligated with the previously gel purified linearized pKP1 vector but still no positive clones were obtained after transformation into competent *E. coli* cells. For further troubleshooting, pure undigested pKP1 plasmid was digested with *Xba*I and *Sac*I, both individually and in combination with *Hind*III, and loaded on a gel to check that the two enzymes actually cut as intended. As can be seen in figure 13, *Xba*I/*Hind*III leaves most of the plasmid uncut indicating that *Xba*I cuts pKP1 poorly for unknown reasons.

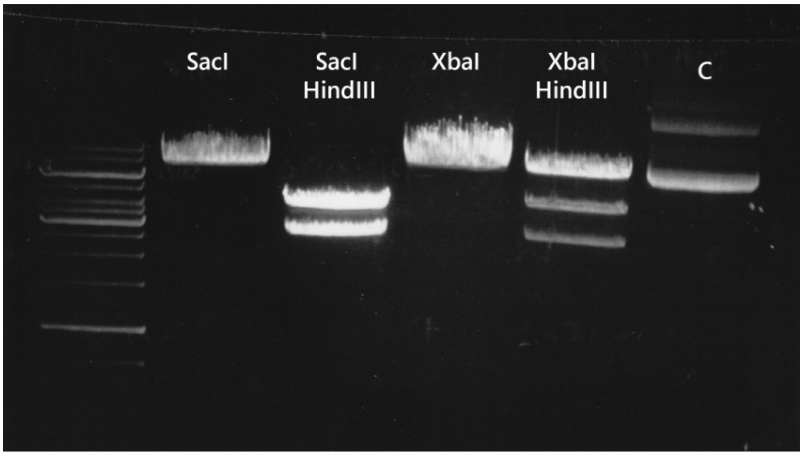

Figure 13. Test digestion of pKP1. The test digest revealed that *Xba*I cut the pKP1 vector poorly. *Xba*I/*Hind*III should give fragment lengths of 2578 and 3978 bp, but had one extra strong band at the same size of uncut pKP1 (C). *Sac*I/*Hind*III gave two clean bands at the correct sizes 2606 and 3950 bp.

Fortunately, there is a recognition site for *Spe*I, producing the same sticky ends as *Xba*I, next to the faulty *Xba*I recognition site in pKP1. The *Spe*I enzyme was tested together with *Eco*RI and *Hind*III and gave results indicating a successful digest (figure 14). The pPK1 plasmid was therefore double digested with *Spe*I/*Sac*I before loaded on a gel allowing extraction of the desired band replacing the *Xba*I/*Sac*I band in the ligation (figure 14).

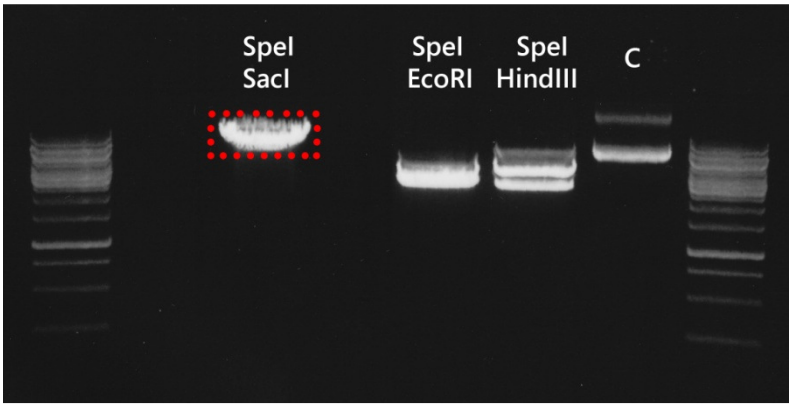

Figure 14. Digestions of pKP1. The *Spe*I/*Eco*RI gave two overlapping bands of the expected fragment lengths 2832 and 3724 bp while *Spe*I/*Hind*III gave two bands of the expected fragment lengths 2572 and 3984 bp. The control (C) was undigested pKP1 vector. The high molecular *Spe*I/*Sac*I digested vector band was extracted for gel purification (marked with dotted red line).

The new gel purified vector pKP1 opened with *Sac*I/*Spe*I (instead of *Sac*I/*Xba*I) was now ligated with the gel purified 3PFR fragment from TOPO clone 1 yielding numerous colonies after transformation. Plasmid DNA was extracted and test digested from 16 clones with the enzymes *Psi*I (figure 15), *Bpu*10I (figure 16) and *Bst*BI (figure 17). Out of 15 plasmids that gave results indicating a successful 3PFR insertion, clone 7 was chosen and given the name pKP2.

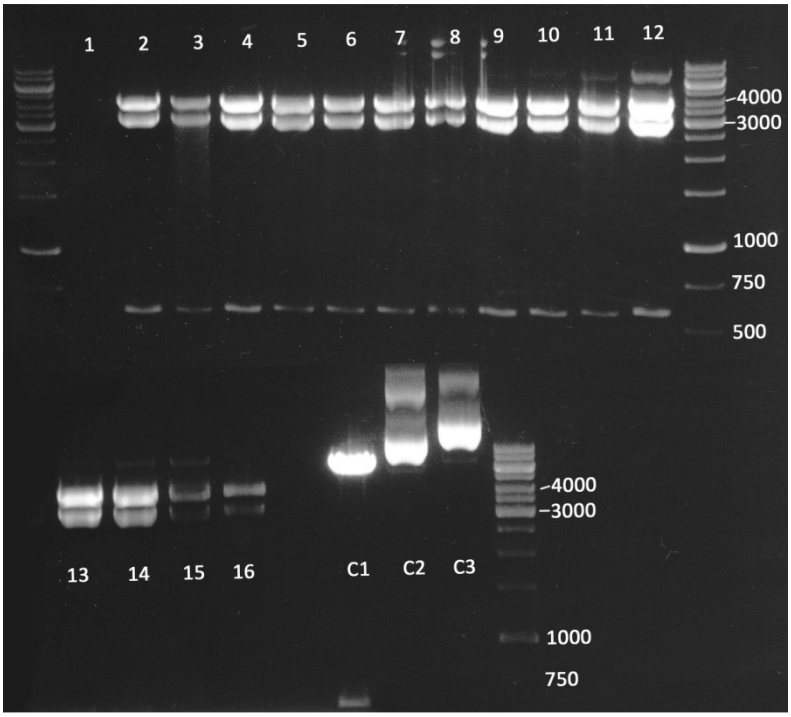

Figure 15. *Psi*I test digestion. The desired ligation product should have bands of 600, 3093 and 3966 bp compared to the digested pKP1 vector (C1) that should have fragment sizes of 600 and 5956 bp. C2: circular empty vector and C3: circular clone 7.

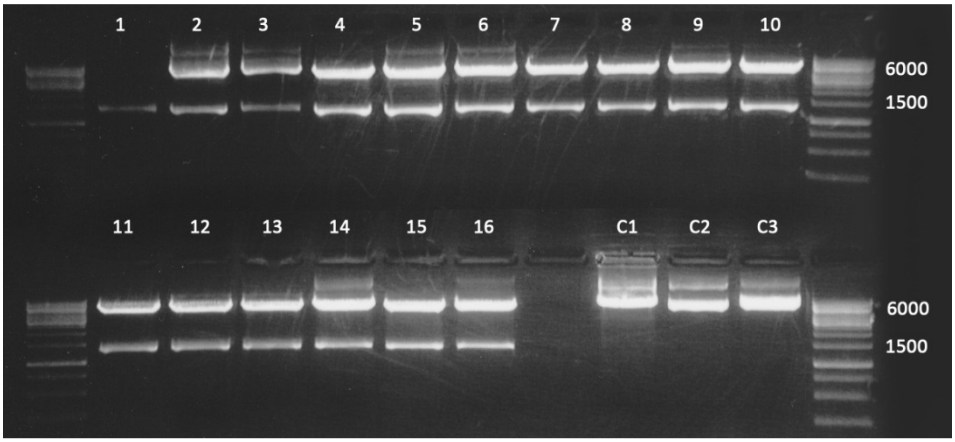

Figure 16. *Bpu*10I test digestion. The desired ligation product should have bands of 1361 and 6298 bp compared to the digested empty vector (C1) with one cut resulting in a linearized fragment of 6556 bp. C2: circular empty vector; C3: circular clone 7.

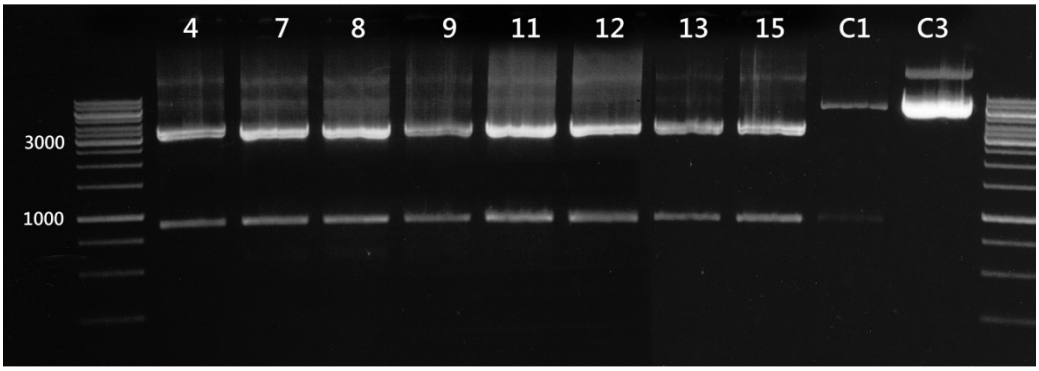

Figure 17. *Bst*BI test digestion. The desired ligation product should have bands of 887, 3219 and 3553 bp compared to the digested empty vector (C1) that have two cuts generating fragment lengths 887 (a weak band in figure) and 5669 bp. C2: circular empty vector; C3: circular clone 7.

5.5 Large scale plasmid preparation and preparative linearization

One bacteria colony containing pKP2, and one containing the *PpMC3* knockout construct plasmid were grown in overnight cultures used for large scale plasmid preparations. The DNA concentration was measured to be 1500 ng/µl for pKP2 and 500 ng/µl for the *PpMC3* knockout construct. The vectors were linearized using restriction enzymes *Sac*I/*Sal*I for pKP2 and *Sac*I/*Kpn*I for the *PpMC3* knockout construct. The products were tested on a gel and the fragment sizes were correct (not shown). The pKP2 plasmid was sent for sequencing using all sequencing primers in list 1. The sequencing results was not obtained before the deadline of the report and will therefore not be discussed further.

5.6 Moss transformation

The two pre-existing knockout lines *Ppmc*1 and *Ppmc2* together with the wild type were transformed with the *PpMC3* and *PpMC5* knockout constructs. As shown in figure 3, the aim was to generate two single knockout lines (*Ppmc3* and *Ppmc5*), and four double knockout lines (*Ppmc1/3*; *Ppmc1/5*; *Ppmc2/3*; *Ppmc2/5*).

The protoplasts that were generated under the transformation procedure looked healthy when counted using a Burcher chamber. The transformed protoplasts grown on regeneration media was studied under microscope after 3 days. The protoplasts had regenerated cell walls and protonemal outgrowth was clearly visible.

After 6 days on media without selection, transformants were transferred to hygromycin plates and grown for 13 days before the regeneration and transformation frequencies were estimated. The transformation frequency was low, with only a few colonies on each of the plates. The frequencies of regeneration and transformation after transformation with the *PpMC3* knockout construct are shown in table 2.

Background	regeneration frequency	Transformation frequency
Wt	0,0385	0,000848
MC1	0,0542	0,000877
MC2	0,0583	0,000657

Table 2. Regeneration and transformation frequency of protoplasts during the transformation with the *PpMC3* knockout construct.

5.7 Genotyping of possible transformants

Due to the time consuming selection of stable transformants, genotyping of possible *Ppmc3* and *Ppmc5* knockouts was not achieved during the short time span of the project. To get an overall picture of all the techniques in the project, genotyping was instead done on putative *Ppatg2* knockout lines (Martin and Thelander, unpublished).

Genomic DNA was extracted using the QuickExtract™ plant DNA Extraction kit (epicentre) and used as the template in PCR reactions with primer pairs 12/13, 14/15 and 16/17 (table 1

and figure 18). Out of 40 extracted DNA samples; none of the PCR reactions gave results for a successful knockout transformation. Primer pair 16/17 amplifying the *Ppatg2* wild type locus gave bands indicating an unsuccessful transformation for the samples tested (not shown).

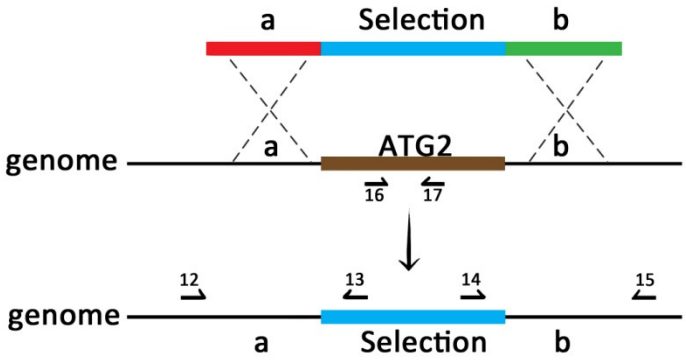

Figure 18. The putative ATG2 knockouts were tested with primer pairs 12/13 and 14/15 for both sides of the selection marker. A negative control with primer pairs 16/17 *Ppatg2* resulted in PCR products of correct size.

6. Discussion

The objective of the project was to produce a *PpMC5* knockout construct to be transformed into *Physcomitrella patens* along with an existing *PpMC3* knockout construct to generate knockout lines. Setbacks during the cloning experiments made the production more complicated than first intended. The goal was achieved through troubleshooting and the knockout experiments were accomplished during the project.

6.1 The demands of a KO construct

P. patens can be transformed at a high frequency with targeted gene replacement, which relies on HR between the ends of knockout construct and the regions flanking the genomic locus to be targeted (Kamisugi et al. 2006).

The high rate of HR that *P. patens* exhibits compared to seed plants is poorly understood (Puchta, 2002). It is possible that proteins involved in the process are different, or that the mechanism is different from other plants. It is known that prokaryotes and lower eukaryotes favor the use of homologous sequence to repair double stranded DNA breaks (Gorbunova and Levy, 1999; Kamisugi et al. 2006).

A construct containing a selectable marker flanked by homologous DNA with an overall homology of 1 kb is enough for a 50 % yield of targeted gene replacement (Kamisugi et al., 2005). However there are examples of insertions that do not result in a complete deletion of the targeted sequence. One of these is when HR occurs in one homologous arm in the construct, and the other arm gets inserted in the breakpoint by non-

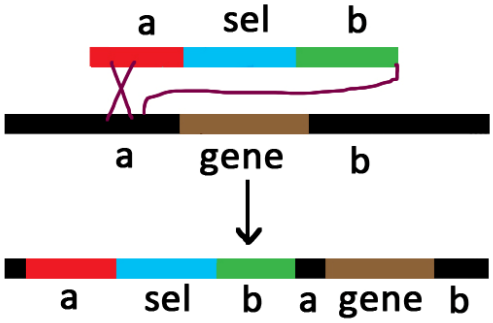

Figure 19. DNA insertion by non-homologous end joining.

homologous end joining (figure 19; Kamisugi et al. 2006). This will produce a transformant that will retain the gene intact, but also survive on selection. The gene targeting construct can also be inserted at regions that are not homologous, this occurs at a lower frequency.

6.2 Selection of stable transformants

The production of stable transformants is time consuming. Transformed protoplasts have to regain their cell walls and start to grow on the plates before they are transferred to selective media. When transferred to selection, most of the colonies die because they do not contain the resistance cassette. Some transformants will retain the resistance gene outside the chromosome when on selection (unstable transformants). Therefore the culture is put off selection, and then back to selection. Most of the unstable transformants will die on the second selection, when the extra-chromosomal resistance gene has not been retained. The true transformants, with the resistance gene inserted into the genome will not be affected by the change of selection.

6.3 Phenotyping and gene redundancy

The presence of multiple copies of metacaspases in the *P. patens* genome makes this type of research a hard task since the possibility that redundant gene function can replace a knocked out gene and conceal the effects of the gene deletion. In the *P. patens* metacaspase family there are two type I and four type II metacaspases (figure 2). The production of the metacaspase type I double knockout line stand a good chance of giving rewarding results as they will result in a complete metacaspase type I knockout line. The metacaspase type II knockout lines produced may well also give informative results, since the two genes in the double knockout are closely related (figure 2). There will still be two type II genes intact in the type II double knockout lines though, and the risk is therefore substantial that the phenotype might not differ from the wild type.

6.4 Reproductive organs

The development of archaegonia has been well characterized (Landberg et al., 2012), and deviations from the wild type will give insight in if the *PpMC3* and *PpMC5* proteins are essential for the clearance of the central cell file. If canal cell PCD is inhibited or delayed in the knockout mutants, it will indicate that metacaspases are involved in the process.

6.5 Cloning problems and Troubleshooting

The building of the *PpMC5* knockout construct was a bigger challenge than expected. The 5PFR insertion into pMT123 vector was successfully achieved without problems. Out of 17 clones, 2 gave positive results for an insertion. The low insertion frequency could be explained by the *Sal*I/*Hind*III digested pMT123 vector not being gel purified; leaving short DNA fragments that can bind back during ligation.

The insertion of the 3PFR involved a lot of work to find the step responsible for the failure of successful insertion. The concentration of the 3PFR was believed to be too low, and the

commercial pCR™II-Blunt-TOPO® cloning vector was used. The TOPO vector has a very high transformation frequency due to the lethal *ccdB* gene that is interrupted by the inserted 3PFR fragment. A TOPO vector that binds back to itself will therefore not produce a surviving bacteria colony. The *Xba*I restriction enzyme showed to be incompetent by test digestion of pKP1 plasmid, leaving most of the sites uncut. This could be because of a point mutation in the pKP1 vector at the particular recognition site. Had this been detected earlier, the construct could have been completed much faster.

The screening methods to find plasmids containing the desired inserts could also have been done with a PCR reaction with specifically designed primers for the flanking regions. The completed construct was sent for sequencing, and will give a final proof that both 5PFR and 3PFR are inserted at the correct place in the pKP2 vector.

6.6 Regeneration/transformation frequency

The transformation frequency during moss transformation is affected by different factors. One of these factors is the quality of the knockout construct, the length of the flanking regions is important. The DNA concentration of the construct is important, if the concentration is too low, the transformation frequency will be lower. If the concentration is too high, a higher risk of insertions at non-targeted genomic regions will be a problem.

The regeneration frequency of the protoplast on the other hand is affected by the quality of the moss starting material. The protoplasts are very sensitive to osmotic pressure and external forces. Handling of the protoplasts carefully is therefore essential for a good regeneration frequency. As seen in table 2, both the regeneration frequency and transformation frequencies are low. A lot of the protoplasts do not survive the process.

6.7 Future perspectives

The *Ppmc3* and *Ppmc5* knockout lines will hopefully show phenotypes that will give insight into the function of the metacaspase gene family. Because of possible gene redundancy, more metacaspase knockout lines could be produced targeting type II genes that have not yet been targeted.

8. Acknowledgements

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Uppsala Biocenter
Plant Biology and Forest Genetics
SLU
PO Box 7080
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